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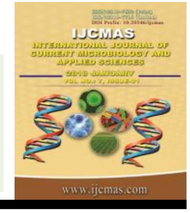

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Green Synthesis of Nanosilver from *Azadirachta indica* Leaves and Its Antibacterial Activity

S. Bharathi[1], D. Saravanan[2*] and M. S. Nalina Sundari[1]

[1]Department of Zoology, Queen Mary's College, Chennai – 600 004, Tamil Nadu, India
[2]Centre for Laboratory Animal Technology and Research, Sathyabama University, Chennai - 600 119, Tamil Nadu, India

**Corresponding author*

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A B S T R A C T

In this study, silver nanoparticles (AgNPs) using leaf extracts of *Azadirachta indica* were synthesized and its antibacterial activity was investigated. The synthesis of AgNPs was done by using the green synthetic method and analyzed by UV-Visible spectroscopy, Fourier transform infrared spectroscopy and transmission electron microscopy. The antibacterial activity of AI AgNPs was studied by agar well diffusion method. The prepared AI AgNPs showed characteristic absorption peak at 440 nm in the UV-Vis spectrum. Fourier transform infrared spectra had shown that the biomolecules present in leaf extract were responsible for the reduction and capping material of silver nanoparticles. Transmission electron microscopy results revealed that the AgNPs were mostly spherical with an average size ranging from 20-50 nm. The AI AgNPs showed good antibacterial activity against *Pseudomonas aeruginosa*, *Escherichia coli*, *Bacillus cereus*, and *Staphylococcus aureus.* In agar well diffusion method, the maximum zone of inhibition was found against *Escherichia coli* with 30 mm and minimum zone of inhibition was found to be against *Bacillus cereus* with 21 mm. The present study explored that *Azadirachta indica* which are efficient producers of AgNPs and could act as safe and cost-effective with potential antibacterial activities. These findings encourage studying AI AgNP further for their potential biological applications.

Introduction

Nanotechnology, a newly evolved discipline aims the creation, manipulation and application of structures in the nanometre size range. Nanotechnology has the potential to revolutionize the pharmaceutical industry with new tools for the molecular treatment of diseases, and rapid disease detection. It advances materials with a nano-dimension provides several means for innovative design of nano-size drug delivery systems (Nano systems) to overcome biological barriers in order to direct the drug. The biological activity of AgNPs depends on factors including surface chemistry, size, size distribution, shape, particle morphology, particle composition, coating/capping, agglomeration, and dissolution rate, particle reactivity in solution, efficiency of ion release, and cell type, and the type of reducing agents used for the synthesis of AgNPs are a crucial factor for the determination of its biological activity (Nikalje, 2015). Silver has a strong antimicrobial potential, which has been used since the ancient times. Antimicrobial effect of silver can be increased by manipulating their size at nano level. Because of their change in physiochemical properties, AgNps have emerged as antimicrobial agents against bacteria, fungi, and virus owing to their high surface-area to volume ratio with unique chemical and physical properties (Kim *et al.,* 2007).

A number of approaches are available for the synthesis of silver nanoparticles for example, reduction in solutions, chemical and photochemical reactions in reverse micelles, thermal decomposition of silver compounds, radiation assisted, electrochemical, sonochemical, microwave assisted process and recently via green chemistry route using various natural products like green tea *Camellia sinensis*, leaf broth natural rubber, starch, Aloe vera plant extract and recently from our laboratory by using lemon grass extract (Salehi, 2016). Synthesis of nanoparticles using biological entities has great interest due to their unusual optical chemical, photoelectro-chemical and electronic properties. The synthesis and assembly of nanoparticles would benefit from the development of clean, nontoxic and environmentally acceptable 'green chemistry' procedure, probably involving organisms ranging from bacteria to fungi and even plants.

Among the various biological sources, plants have vast potentiality and this work aims to apply a biological green technique for the synthesis of silver nanoparticles. In this regard, leaf extract of *Azadirachta indica* (commonly known as neem) a species of family Meliaceae was used for bioconversion of silver ions to nanoparticles. This plant is commonly available in India and each part of this tree has been used as a household remedy against various human ailments from antiquity and for treatment against viral, bacterial and fungal infections (Omoja *et al.*, 2011).

Materials and Methods

Selection and collection of plant material

A. indica leaf extract was used to prepare silver nanoparticles on the basis of cost effectiveness, ease of availability and its medicinal property. Fresh leaves were collected from Tamil Nadu Veterinary and Animal Sciences University, Kattangulathur, Chennai. They were surface cleaned with running tap water to remove debris and other contaminated organic contents, followed by double distilled water and air dried at room temperature. About 20 gm of finely cut leaves were kept in a beaker containing 200 ml double distilled water and boiled for 30 min. The extract was cooled down and filtered with Whatman filter paper no.1 and extract was stored at 4°C for further use (Suresh *et al.*, 2014).

Biosynthesis of silver nanoparticles

For the biosynthesis of silver nanoparticles, 1.5 ml leaf extract was mixed with 30 ml of 1mm aqueous silver nitrate solution and incubated at room temperature in dark condition for 24 hours. The bio-reduction of silver ions in the solution was monitored using UV-Visible spectroscopy. The formation of a reddish brown-coloured solution indicated the formation of the silver nanoparticles. The silver nanoparticles obtained from the solution were purified by repeated centrifugation at 12,000 rpm for 20 min followed by dispersion of the pellet in sterile deionized water three times to remove the water-soluble biomolecules such as proteins and secondary metabolites. The water suspended nanoparticles were lyophilized and freeze dried silver nanoparticles were used to characterize the structure and composition (He *et al.,* 2017).

Characterization of synthesized silver nanoparticles

UV-Vis spectral analysis

The bioreduced aqueous extract of *Azadirachta indica* containing silver nanoparticle was confirmed by taking an aliquot of 100µl of the extract for measuring the absorbance. The absorption maximum was scanned by UV-Vis spectrophotometer at the wavelength of 300- 800 nm using Schimadzu UV- vis spectrophotometer

Fourier transform infra-red analysis

In Fourier Transform Infrared spectroscopy (FT-IR), IR radiation is passed through a sample. Some of the infrared radiation is absorbed by the sample and some of it is passed through the sample or transmitted. The resulting spectrum represents the molecular absorption and transmission, creating a molecular fingerprint of the sample. Elemental analysis of the synthesized silver nanoparticles was studied by FT-IR.

TEM analysis

The 200 kV ultra-high-resolution transmission electron microscope (TEM) was used to visualize the morphology of the Ag NPs. TEM grids were prepared by placing a 5 µL of the AgNP solutions on carbon-coated copper grids and drying under lamp.

Antimicrobial activity Silver nano particles

Test organisms *Staphylococcus aureus* (ATCC 6538), *Bacillus cereus* (NCIM 2106), *Escherichia coli* (ATCC 8739), *Pseudomonas aeruginosa* (ATCC 9027) were obtained from Centre for Laboratory Animal Technology and Research, Sathyabama University, Chennai. Each test strain was inoculated in Mueller Hinton liquid medium (5ml broth) and incubated in a temperature controlled shaker (120 rpm) at 37°C for 18 hours.

Well diffusion assay

Bacterial growth with 0.5 McFarland standard was inoculated into nutrient agar plates using sterile cotton swab. About 5 mm size well was made and different concentrations 50, 75, 100 µl of AI AgNp were added into it. Sterile 1mM $AgNO_3$ solution was used as blank exhibited no activity against any of the used organisms. Amoxycillin (10µg) was used as standard drug (CLSI, 2005).

The plates were incubated at 37°C for 24 hours, and the zone of inhibition (ZOI; mm) appearing around the wells was recorded All the plates were observed for zone of inhibition after incubation at 37°C for 24 hours (Kumar *et al.,* 2017).

Results and Discussion

Synthesis of Silver nanoparticles

Reduction of silver ions into silver nanoparticles during exposure to plant extracts was observed as a result of the color change. The color change is due to the Surface Plasmon Resonance phenomenon. The aqueous silver ions were reduced to silver nanoparticles when added to natural plant extract of *A.indica*.

It was observed that the color of the solution turned from yellow to bright yellow and then to dark brown within 2 hours of the reaction, which indicated the formation of silver nanoparticles. The formation and stability of the reduced silver nanoparticles in the colloidal solution was monitored by UV–vis spectrophotometer analysis.

UV analysis

In UV spectroscopy, AgNPs give a characteristic absorbance band due to the excitation mode of their surface plasmons which depend on the nano particle size. In the present work, the AgNPs are rapidly formed after 2h incubation period by the aqueous extract as evident from the appearance of brownish yellow color and λmax appeared at 440 nm as depicted in Figure 1.

The obtained results also correlate with that of previous works done (Edison and Sethuraman 2012) by which the silver nanoparticles synthesized by using plant fruit aqueous extract.

Yang and Li (2013) carried out investigations on biosynthesized silver nanoparticles shown the in UV spectrum (412 to 434 nm) and hence strongly support the present results.

It is known that an absorption band appears at about 400–440 nm because of the surface plasmon resonance in silver nanoparticles (Pastoriza-Santos and Liz-Marzaın, 2008).

FTIR analysis

The IR spectra provided information about the local molecular environment of the organic molecules on the surface of nanoparticle. In the present work, FTIR spectral measurements were carried out to identify the potential biomolecules in *A. indica* leaf extract which is responsible for reducing and capping the bioreduced silver nanoparticles. FTIR can be used to analyze a wide range of materials in bulk or thin films, liquids, solids, pastes, powders, fibres, and other forms. FTIR analysis can give not only qualitative (identification) analysis of materials, but, with relevant standards, can be used for quantitative (amount) analysis. FTIR measurements were carried out to identify the possible biomolecules responsible for capping and efficient stabilization of the metal nanoparticles synthesized by *A. indica* leaf extract. The results of FTIR analysis of this study show different stretches of bonds shown at different peaks; 3432.94—N–H stretch, 2777.28—single aldehyde, 2676.19—C–H; O–H, 2071.75—C≡C, 1637.58—C=C, and 1121.56—C=O (Figure 2).

Fig.1 UV- Spectral analysis of biosynthesized AI AgNp

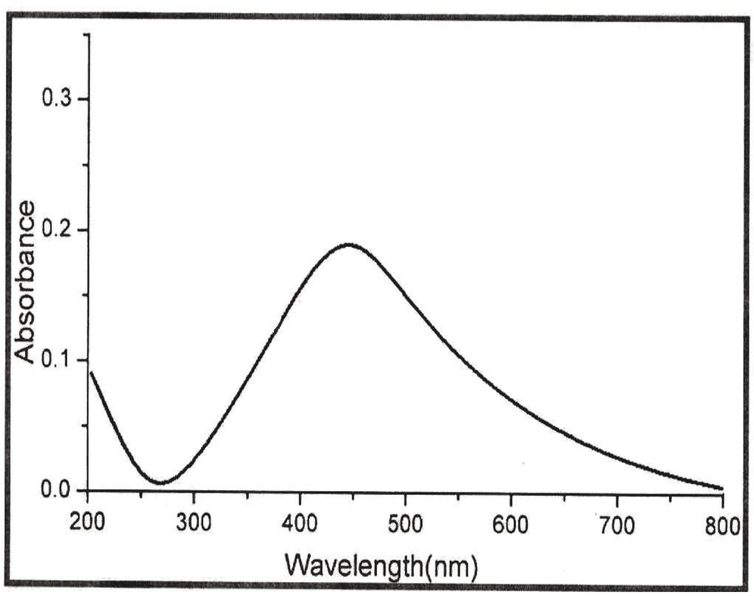


Fig.2 FTIR analysis of AI

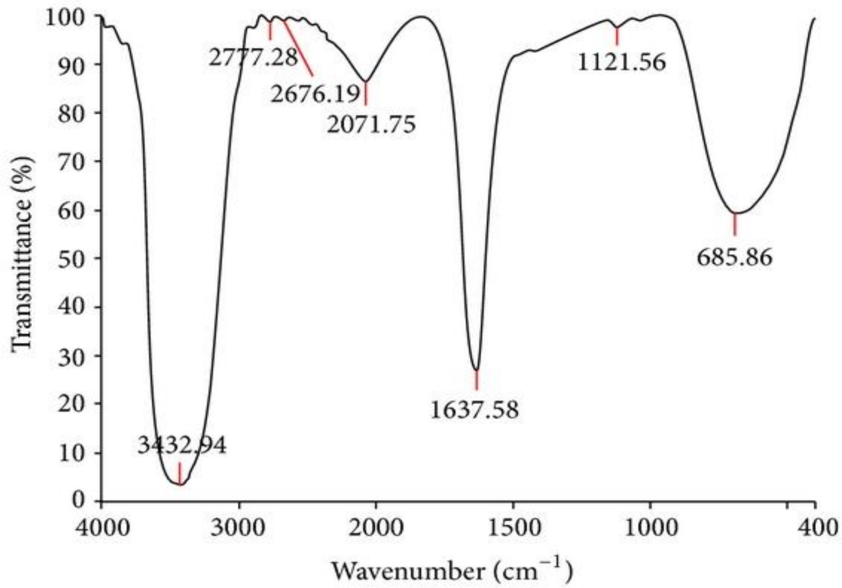
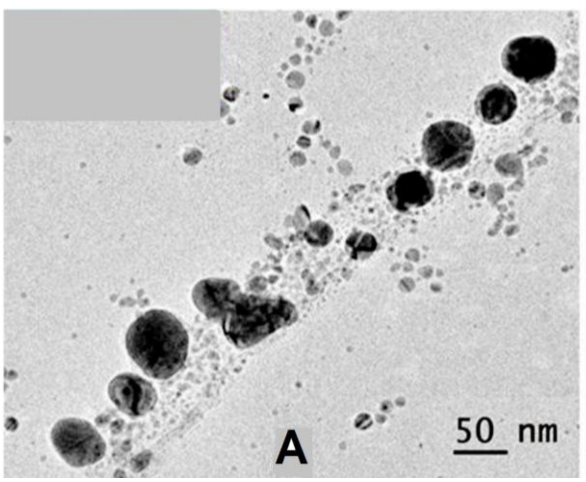


Fig.3 TEM images of silver nanoparticles. Formed by the reaction of 1 mM silver nitrate and 5% leaf extract of *A. indica* a) 50nm, b) 20nm



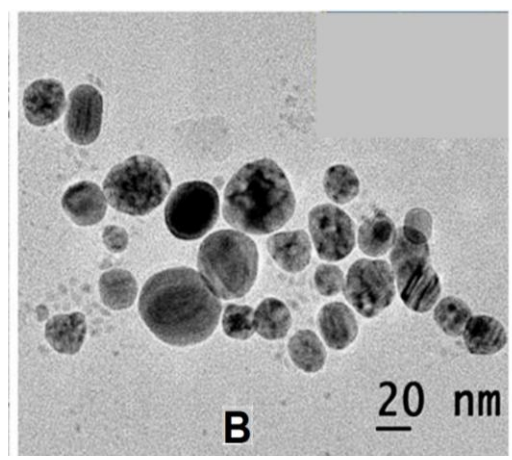


Table.1 Zone of inhibition produced by AgNps (Plant extract), reference antibiotic Rifamycin

S. No.	Test Organisms	Zone of inhibition in mm			Rifampicin
		50 µl	75 µl	100 µl	50µg/ml
1.	*Staphylococcus aureus,*	12	19	25	16
2.	*Bacillus cereus*	14	18	21	18
3.	*Escherichia coli*	20	25	30	20
5.	*Pseudomonas aeruginosa,*	15	17	22	24

Transmission Electron Microscopy (TEM) analysis

TEM images clearly shows that the AI AgNp were spherical in shape with the size ranging from 20- 50nm (figure 3)

Antimicrobial activity of AI silver nanoparticles

The antimicrobial activity of silver nanoparticles synthesized by natural A. indica leaf extract was investigated against various pathogenic organisms such as *S. aureus, P. aeruginosa, E. coli and B. cereus* using well diffusion method. The diameter of inhibition zones (mm) around each well with silver nanoparticles solution is represented in Table 1. In agar well diffusion method, the maximum zone of inhibition was found against *Escherichia coli* with 30 mm and minimum zone of inhibition was found to be against *Bacillus cereus* with 21 mm. The silver nanoparticles showed efficient antimicrobial property compared to reference antibiotic (Rifamycin).

The nanoparticles get attached to the cell membrane and also penetrated inside the bacteria. The bacterial membrane contains sulfur containing proteins and the silver nanoparticles interact with these proteins in the cell as well as with the phosphorus containing compounds like DNA. The nanoparticles preferably attack the respiratory chain, cell division finally leading to cell death. The nanoparticles release silver ions in the bacterial cells, which enhance their bactericidal activity (Feng *et al.*, 2000).

The silver nanoparticles have been produced by *A. indica* extracts, which is an efficient and eco-friendly process. UV–vis spectrophotometer, FTIR and SEM techniques have confirmed the reduction of silver nitrate to silver nanoparticles. The zones of inhibition were formed in the antimicrobial screening test indicated, that the Ag NPs synthesized in this process has the efficient antimicrobial activity against pathogenic bacteria. The biologically synthesized silver nanoparticles could be of immense use in medical field for their efficient antimicrobial function.

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